Aquatic Toxicology 91 (2009) 161–170

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Comparative susceptibility to atrazine of three developmental stages of *Rhinella arenarum* and influence on metamorphosis: Non-monotonous acceleration of the time to climax and delayed tail resorption

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ARTICLE INFO

Article history:
Received 28 May 2008
Received in revised form 2 July 2008
Accepted 3 July 2008

Keywords:
Atrazine
Amphibian
Metamorphosis
Non-monotonous dose–response
Endocrine disruption
U-shaped

ABSTRACT

Acute and subchronic toxicity of atrazine was evaluated in embryos (stage 4) and in premetamorphosis (stage 25) and prometamorphosis (stage 38–39) larvae of the common South American toad *Rhinella arenarum* (Anura: bufonidae). The influence of atrazine on the last stages of metamorphosis was also examined by exposing prometamorphosis larvae until completion of metamorphosis. Results obtained revealed that larvae in premetamorphosis are more sensitive than larvae in prometamorphosis and that these are, in turn, more sensitive than embryonic stages. Indeed, concentrations of atrazine as high as 30 mg/L had little effects on embryonic stages, the embryos surviving and developing in a similar manner as controls. LC50s of premetamorphosis larvae equaled 27.16, 7.03 and 2.32 mg/L of atrazine after 4, 14 and 21 days of exposure, respectively, compared to LC50s values of 18.27 and 14.43 mg/L after 14 and 21 days of exposure for larvae in prometamorphosis. In experiments with premetamorphosis larvae, the range of tested concentrations was extended to very low concentrations (down to 0.0001 mg/L) to examine whether recent findings of greater mortality at lower doses than at higher doses were also observed in *R. arenarum* but no such pattern was found. Exposure of prometamorphosis larvae to concentrations of atrazine of 10 mg/L and above widely prevented completion of metamorphosis and caused important mortality. Alternatively, whereas all animals eventually completed metamorphosis when exposed to concentrations of atrazine between 0.1 and 5 mg/L, the timings of metamorphosis were altered starting from 0.1 mg/L, the lowest concentration tested. Indeed, a significant decrease in the time needed for 50% of the larvae to reach the metamorphic climax (stage 42) was observed within this range of atrazine concentrations, the response presenting a U-shaped non-monotonic dose–response curve. Larvae exposed to these concentrations of atrazine also needed significantly more time for completing tail resorption, this effect being equivalent at all concentrations. Overall, the combination of these two different facets of atrazine influence on metamorphosis resulted in a significant acceleration of metamorphosis at 1 mg/L and a significant increase in the duration of metamorphosis at 5 mg/L, whereas no significant difference was observed with 0.1 mg/L.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) has been a major agricultural herbicide for more than 40 years. Initially registered in 1958, it selectively controls broadleaf and certain grass weeds by inhibiting photosynthesis. It is primarily used on corn, sorghum, and sugarcane and can be applied to crops preplant, pre-emergence, or post-emergence. Selective control means that the target weeds are controlled, with little or no injury to the crop. Indeed, atrazine is well tolerated by actively growing corn or sorghum, which absorb and metabolize the herbicide, thus deactivating it. These factors and atrazine's relatively low cost have contributed to make it an extremely popular herbicide (Ribaudo and Bouzaher, 1994).

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doi:10.1016/j.aquatox.2008.07.003

In recent years, however, the widespread use of atrazine has raised major concerns for a number of reasons including its presence in drinking water, links to cancer and Parkinson's disease in humans, and adverse effects on aquatic organisms (PAN, 2002). These concerns triggered the initiation in 1994 of a Special Review of the herbicide by the U.S. Environmental Protection Agency (EPA), which approved its continued use in October 2003. Paradoxically, that same month the European Union (EU), which operates under the precautionary principle, announced a ban of atrazine because of ubiquitous and unpreventable water contamination (Sass and Colangelo, 2006).

The widespread use of atrazine combined with its moderate solubility in water (33 mg/L at 22 °C) and long half-life (between 1.73 and 740 days) indeed make it a common contaminant of surface and subterranean waters (Solomon et al., 1996). In agricultural areas, rates of detection are often near 100% (Frey, 2001; Giroux, 2002) with concentrations in rivers and streams in the parts per billion range (usually below 0.02 mg/L). It is common, however, for the first few rain events after an application to observe runoff with elevated concentrations of atrazine that may reach the parts per million level (Kadoum and Mock, 1978; Klaine et al., 1988; Eisler, 1989; Huber, 1993; Battaglin et al., 2000). These peaks of atrazine-containing runoffs normally occur in springtime when application is maximal, and are particularly contaminating for shallow ponds adjacent to agricultural fields. As these sites are used for amphibian breeding at this same time of year, the possibility of negative impacts on amphibian reproduction and larval development has been raised (Howe et al., 1998; Allran and Karasov, 2000).

As a result, several studies have been conducted in the past few years to examine the potential influence of atrazine on larval development; the effects most frequently reported including alterations of growth, metamorphosis and gonad differentiation. Some studies described hermaphroditism and single-sex polygonadism in *Xenopus laevis* and *Rana pipiens* from concentrations as low as 0.0001 mg/L (Hayes et al., 2002a,b,c, 2006), even though other studies on the same species and on *Rana clamitans* failed to detect any such effects (Coady et al., 2004; Orton et al., 2006; Jooste et al., 2005; Oka et al., 2008), or required slightly higher concentrations (0.025 mg/L) for hermaphroditism to be observed (Carr et al., 2003). In addition, Tavera-Mendoza et al. (2002a,b) demonstrated that a short exposure (48 h) to 0.021 mg/L of atrazine is sufficient for significantly reducing the pool of primordial germ cells in both the testis and the ovaries.

With respect to growth, exposures to low environmentally relevant concentrations have generally failed to detect any effect (Carr et al., 2003; Coady et al., 2004; Orton et al., 2006) whereas higher concentrations of atrazine have been shown to decrease snout-vent length and body weight (Brown Sullivan and Spence, 2003; Diana et al., 2000). Regarding metamorphosis, a majority of studies have used *X. laevis*. Amongst these, some reported a delay in metamorphosis (Brown Sullivan and Spence, 2003; Freeman and Rayburn, 2005) whereas others, generally the ones using lower concentrations, did not observe any effect (Carr et al., 2003; Oka et al., 2008). As regards other species, various studies have reported non-monotonous effects of atrazine on the time needed for completing metamorphosis. Diana et al. (2000) showed that 0.2 mg/L of atrazine accelerated completion of metamorphosis in *Hyla versicolor* whereas larvae exposed to 2 mg/L did not significantly differ from controls. Similarly, Freeman et al. (2005) also observed an acceleration of metamorphosis in *B. americanus* exposed to 0.25, 0.5 and 1 mg/L of atrazine whereas no difference existed between controls and larvae exposed to 5 and 10 mg/L. On the opposite, Coady et al. (2004) found that metamorphosis was extended with 0.01 but not at 0.025 mg/L of atrazine in *R. clamitans*.

Although little mentioned in the atrazine debate, Argentina is an important user of the herbicide. The country is the second largest exporter of both corn and sorghum with 4 million hectares planted with corn and 820 thousand hectares planted with sorghum in 2007–2008 (SAGPyA, 2008). The vast majority of the grain production originates from a region referred to as the Argentine corn/soy belt, which lies in the humid Pampa. Apart from the longer frost-free period, this belt resembles the U.S. corn belt with similar growing-season temperature and precipitation. As is the case in the U.S., a majority of the corn and sorghum lots receive applications of atrazine as the herbicide is allowed for use without any restriction on cereal and forage crops (Subsecretaria de Recursos Hídricos de la Nación, 2003). Between 1999 and 2006, atrazine use almost doubled in Argentina increasing from 4.9 to 8.7 million liters per year (C.A.S.A.F.E, 2008). Together with an increase in the area dedicated to corn crops, this increase in the use of atrazine is also due to the increased popularity of no-tillage systems in which weeds are controlled chemically rather than mechanically (A.A.P.R.E.S.I.D, 2008). The increasing world demand for corn, which is associated to the expansion of the ethanol industry and of the world's livestock herds, do not let anticipate any imminent change in the situation.

Despite the widespread use of atrazine in the argentine corn/soy belt, very few toxicological studies have been conducted on its autochthonous species and no data are available regarding its diverse and specific amphibian fauna. The aim of the current study was to evaluate the toxicity of atrazine to developmental stages of the common South American toad, *Rhinella arenarum* (Hensel, 1867), an anuran species with a wide distribution in Argentina. For many years and until 2006, *R. arenarum* was called *Bufo arenarum*. However, the species saw its name changed on two occasions in the last few years, first to *Chaunus arenarum* and then to *R. arenarum* (Frost et al., 2006; Chaparro et al., 2007). As an adult, *R. arenarum* is terrestrial and reproduces in shallow temporary ponds that form after heavy rains in the Pampean pastures. Adults normally congregate in large breeding groups and each egg mass can contain as much as 40 thousand eggs (Natale, 2006). Tadpoles form loosely aggregated schools in shallow edges of ponds or on the pond bottom but rarely occur in midwater (Caldwell, 1989; Kehr, 1994). It is common for the water level of the breeding ponds to decrease abruptly after a few days, leaving the surviving larvae experiencing great densities (Natale, 2006).

Acute and subchronic toxicity of atrazine was determined and compared in embryos (stage 4) and in premetamorphosis (stage 25) and prometamorphosis (stage 38–39) larvae of *R. arenarum*. The range of tested concentrations was purposely expanded to very low concentrations in experiments with premetamorphosis larvae in order to examine recent claims of non-monotonic survivorship patterns (Storrs and Kiesecker, 2004). Furthermore, the influence of atrazine on the last stages of metamorphosis was studied by exposing prometamorphosis larvae until completion of metamorphosis.

2. Materials and methods

2.1. Embryos

Adults of the common South American toad, *R. arenarum* (Hensel, 1867), weighing approximately 200–250 g were obtained from a local provider. Ovulation of female toad was induced by means of an intraperitoneal injection of homologous hypophysis suspended in 1 mL of AMPHITOX solution (AS) (Herkovits and Perez-Coll, 2003). Oocytes were fertilized *in vitro* using fresh sperm suspended in AS. The resulting embryos were maintained in AS at 20 ± 2 °C until reaching the stage (Gosner, 1960) required by each

Table 1
Actual concentrations of atrazine measured in experimental solutions (mean ± S.E., $n = 2$)

Nominal concentration (mg/L)	Measured concentration (mg/L)
0	<D.L.
1	1.3 ± 0.4
2.5	2.2 ± 0.37
5	3.9 ± 1.4
7.5	6.0 ± 1.3
10	10.4 ± 1.2
15	13.8 ± 0.4
20	18.9 ± 0.4
25	21.5 ± 1.7
30	25.9 ± 1.6

<D.L. = Below detection limit of 0.02 mg/L.

experimental protocol. Larvae were offered boiled swiss chard *ad libitum* when they began feeding at stage 24–25.

2.2. Preparation of test solutions

Technical-grade atrazine (CAS No. 1912-24-9) with a purity of 98% was obtained from Chem Service (West Chester, PA, USA). Test solutions between 10 and 30 mg/L were prepared by directly weighing and dissolving the corresponding mass of atrazine into one liter of AS. Test solutions of 2.5, 5, and 7.5 mg/L of atrazine were prepared by dissolving a solution of 15 mg/L of atrazine with AS. Test solutions of 1, 0.1, 0.01, 0.001 and 0.0001 mg/L of atrazine were prepared by sequentially diluting a solution of 10 mg/L of atrazine with AS. Because the highest concentration of atrazine tested (30 mg/L) was near the water solubility value (33 mg/L at 22 °C) and precipitation was sometimes observed, 0.2% of acetone were added to the test solutions to insure homogenous dissolution. Each experimental design accordingly included, together with a control group exposed to AS only, a solvent control group that was exposed to AS containing 0.2% of acetone. Atrazine concentrations in test solutions were verified by high-performance liquid chromatography/mass spectrometry. Analytical data obtained were near the nominal values. These data are shown in Table 1.

2.3. Experimental protocols

2.3.1. Subchronic exposures starting with embryos or premetamorphosis larvae

Prior to experiments with embryos, the jelly coat of recently fertilized eggs was dissolved by immersing egg ribbons into a solution of 2.5% thioglycolic acid at pH 7.2. This procedure was not necessary for experiments with premetamorphosis larvae, as these are freely swimming animals. For each experimental treatment, ten embryos (stage 4) or larvae (stage 25) were placed in triplicate 10 cm-diameter glass Petri dishes containing 40 mL of AS with or without atrazine (controls). Nominal concentrations of atrazine tested were 1, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 mg/L. Test solutions were entirely replaced every 48 h, and temperature was maintained between 20 ± 2 °C throughout the experiments which lasted 21 days. Dead embryos and larvae were removed and survival was evaluated every other day. When feeding larvae (stage 24–25 and above) were present in the test Petri dishes, a piece of boiled Swiss chard of approximately 2 cm^2 was added to the test vessels after changing the solution. The experiment using premetamorphosis larvae was repeated four times, larvae from a distinct pair of parents being used on each occasion. Likewise, the experiment starting with embryos was repeated twice, both times with embryos from a distinct pair of parents.

As a previous study by Storrs and Kiesecker (2004) reported atrazine to cause greater mortality at lower doses than higher doses in a number of amphibian species, a second set of 21-day exposure was also conducted with premetamorphosis larvae (stage 25) using a range of concentrations extending four orders of magnitude below the lowest concentration used in the first set of exposures. Atrazine concentrations tested in this second set of exposures were: 0.0001, 0.001, 0.01, 0.1, and 1 mg/L. This experiment was conducted as described above and was repeated four times, larvae from a distinct pair of parents being used on each occasion.

2.3.2. Exposure from prometamorphosis until completion of metamorphosis

Larvae having reached stage 38–39 (two completed back legs) were exposed to the following concentrations of atrazine until completion of metamorphosis: 0.1, 1, 5, 10, 15 and 20 mg/L. Ten larvae were placed in triplicate 20 cm-diameter glass Petri dishes containing 100 mL of AS with or without atrazine (controls). Test solutions were entirely replaced every 48 h, and temperature was maintained between 20 ± 2 °C throughout the experiment. Even though feeding is normally interrupted in metamorphosing tadpoles as mouth and intestine are restructured, a piece of boiled Swiss chard of approximately 2 cm^2 was added to the test vessels after each change of solution to insure food availability. Dead embryos were removed and survival and Gosner stage were evaluated every day. This experiment was executed once with larvae from a single pair of parents.

2.4. Data analysis

For each exposure, the control and solvent control groups were first compared by a *t*-test or by a rank sum test, if normality and equal variance could not be obtained. As differences were never observed between the two control groups, data from both groups were combined for further analyses. The concentrations of atrazine resulting in the mortality of 10, 50 and 90% (LC10, LC50 and LC90, respectively) of embryos and larvae after diverse exposure durations were calculated by fitting a four-parameter logistic regression equation to the survival data using the GraphPad Prism software version 3.02. The LC50 values and their associated standard error were used in a *t*-test to compare data obtained after given intervals of exposures from stage 25 to the corresponding interval when expressing durations of exposure from stage 4 in terms of the days since reaching stage 25. The number of days of exposure from stage 4 was transformed into the number of days of exposure since reaching stage 25 by subtracting 8 days to every interval, which corresponds to the number of days needed by the slowest embryos to reach stage 25. Percentages of embryos presenting malformation in each treatment groups after exposure to atrazine for 4 and 7 days from stage 4 were calculated based on the number of live individuals remaining. The percentages of malformation calculated were compared using a two-way analysis of variance (ANOVA) after confirming normality and equal variance of the data.

In the experiment starting with prometamorphosis larvae, a four-parameter logistic regression equation was fitted for each treatment to the cumulative numbers of animals reaching stage 42 or completing metamorphosis in function of exposure duration using GraphPad Prism software version 3.02. With each curve fitted, the software calculates the time for 50% of the individuals to reach stage 42 (T42) or complete metamorphosis (TCM). The T42 and TCM calculated for each treatment were compared by a one-way ANOVA followed by a Holm-Sidak test for multiple comparisons. The time intervals between T42 and TCM were also calculated by subtracting the value of T42 from the value of TCM obtained for each replicate of each treatment. These values were afterwards compared by a one-way ANOVA followed by a Holm-Sidak test for multiple com-

Table 2
Lethal concentrations 10, 50 and 90 calculated for *R. arenarum* exposed to atrazine from stage 4, 25, or 38–39

Days of exposure	Exposure from stage 4		
	LC10 (mg/L)	LC50 (mg/L)	LC90 (mg/L)
10	15.31 (11.64–20.14)	25.37 (22.86–28.15)	N.A.
14	7.84 (6.02–10.21)	14.41 (12.79–16.23)	26.42 (20.99–33.34)
21	3.51 (2.56–4.81)	7.15 (6.31–8.09)	14.55 (11.12–19.05)
Days of exposure	Exposure from stage 25		
	LC10 (mg/L)	LC50 (mg/L)	LC90 (mg/L)
2	25.76 (22.08–30.13)	N.A.	N.A.
4	18.28 (16.18–20.65)	27.16 (26.01–28.35)	N.A.
7	13.61 (11.86–13.63)	20.11 (18.99–21.3)	29.72 (26.30–33.57)
10	8.04 (6.48–9.98)	14.48 (13.14–15.97)	26.06 (21.58–31.48)
14	3.36 (2.75–4.12)	7.03 (6.48–7.62)	14.69 (12.36–17.46)
21	0.71 (0.31–1.61)	2.32 (1.71–3.17)	7.59 (4.85–11.9)
Days of exposure	Exposure from stage 38–39		
	LC10 (mg/L)	LC50 (mg/L)	LC90 (mg/L)
7	16.26 (15.0–17.66)	N.A.	N.A.
14	12.65 (11.17–14.32)	18.27 (17.38–19.20)	N.A.
21	11.51 (10.45–12.71)	14.43 (14.05–14.83)	18.07 (16.87–19.41)
28	10.02 (9.0–11.14)	12.63 (11.89–13.42)	15.92 (14.72–17.26)

Confidence intervals 95% (CI) are indicated in parenthesis. Values could only be calculated for exposure durations where at least one of the tested concentrations generated a mortality of more than 10%.
N.A. Not available because calculated value is outside the range of tested concentrations.

parisons. All *t*-tests, ANOVAs and multiple comparison tests were conducted using SigmaStat 3.11 statistical software (SPSS, Chicago, IL, USA).

3. Results

3.1. Subchronic exposures starting with embryos or premetamorphosis larvae

When embryos were exposed from stage 4, 10 days of exposure were necessary before a significant decrease in survival could be observed at 25 and 30 mg/L, the two highest concentrations of atrazine tested (Fig. 1a). Mortality thereafter increased steadily in embryos exposed to 7.5 mg/L or more of atrazine, the concentrations necessary for reducing survival to 50% (LC50) equaling 25.37, 14.41, and 7.15 mg/L after 10, 14, and 21 days of exposure, respectively (Table 2). The incidence of malformations observed in embryos was not significantly different from the controls at any of the concentration of atrazine tested (data not shown). Table 3 shows that progression through Gosner stages was similar amongst treatment groups. The only notable differences were that larvae exposed to 1 and 2.5 mg/L of atrazine took a day less than controls to pass from stage 23 to stage 25 whereas groups exposed to 15 mg/L of atrazine or more slightly lagged behind controls from day 4 onward. All animals had reached stage 25 after 8 days of exposure. They remained at stage 25–26 until the end of the 21-day exposure.

Compared to embryos, larvae in premetamorphosis were more sensitive to atrazine and 4 days of exposure were sufficient for significantly reducing survival to 78.3 and 31.8% with 20 and 30 mg/L of atrazine, respectively (Fig. 1b). The concentration of atrazine required for reducing survival to 50% thereafter decreased steadily with exposure duration, the LC50s equaling 27.16, 20.11, 14.48, 7.03 and 2.32 after 4, 7, 10, 14 and 21 days of exposure, respectively (Table 2). These LC50 values represent 57, 49 and 33% of the LC50 values obtained when exposing embryos from stage 4 for 10, 14 and 21 days, respectively, demonstrating a greater resistance of the earlier stages. As a previous study by Storrs and Kiesecker (2004) reported atrazine to cause greater mortality at lower doses than

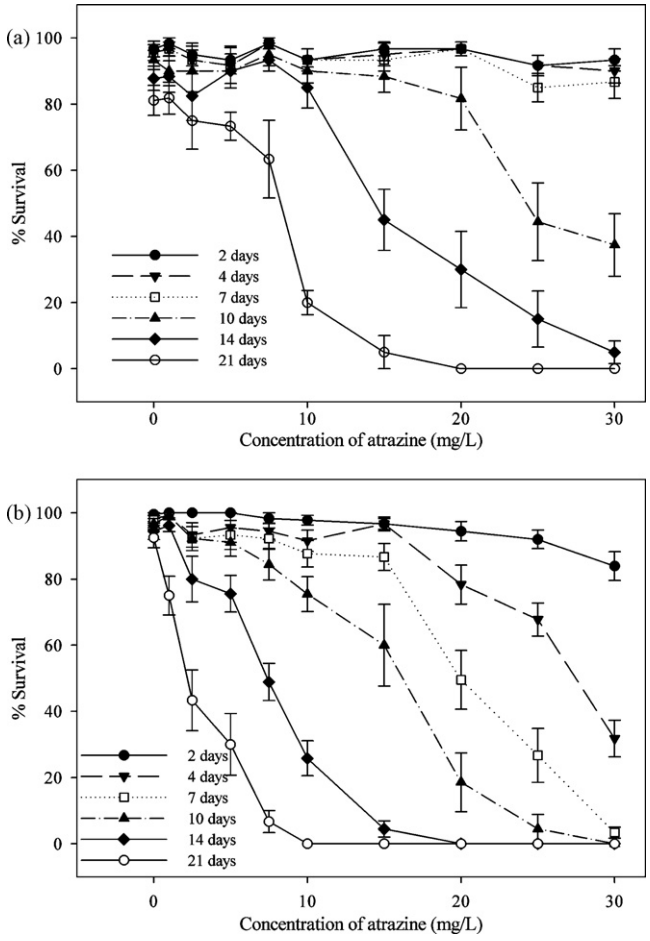

Fig. 1. Concentration–response curves describing the survival of *R. arenarum* in function of atrazine concentration when exposed from (a) stage 4 and (b) stage 25 for 2, 4, 7, 10, 14 and 21 days.

Table 3
Progression through Gosner stages until reaching stage 25 of embryos of *R. arenarum* exposed to atrazine from stage 4

Days	C	At 1	At 2.5	At 5	At 7.5	At 10	At 15	At 20	At 25	At 30
0	4	4	4	4	4	4	4	4	4	4
1	12	12	12	12	12	12	12	12	12	12
2	17	17	17	17	17	17	17	17	17	17
3	18	18	18	18	18	18	18	18-	18-	18-
4	20	20	20	20	20	20-	20-	20-	19	19
5	23	23	23	23	23	23	23-	23-	23-	23-
6	24	25	25	24	24	24	24	23	23	23
7	25	25	25	25	25	25	24	24	24	24
8	25	25	25	25	25	25	25	25	25	25

The number of the Gosner stage reached by the embryos after a given number of days of exposure are indicated. The number at the side of the abbreviation "At" represents the concentration of atrazine in mg/L. Controls are denoted by C. As all individuals of a given treatment normally evolved through Gosner stages at the same time, standard errors could not be calculated. A minus sign next to a Gosner stage number indicates embryos presenting an early version of the given stage.

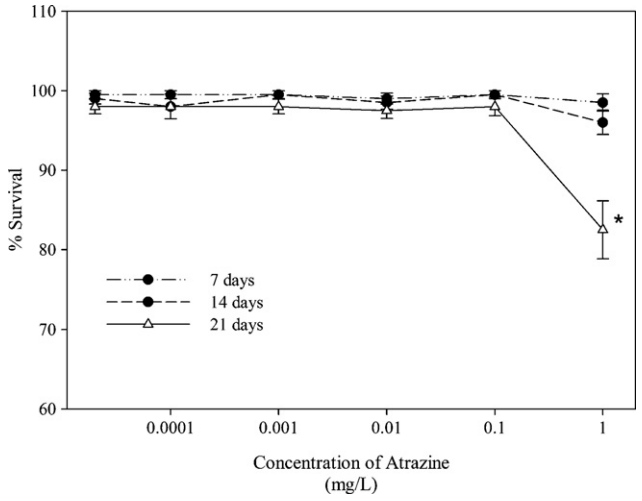

Fig. 2. Concentration–response curves describing the survival of larvae of *R. arenarum* when exposed from stage 25 to low concentrations of atrazine for 7, 14 and 21 days. * = significantly different from controls ($p < 0.05$).

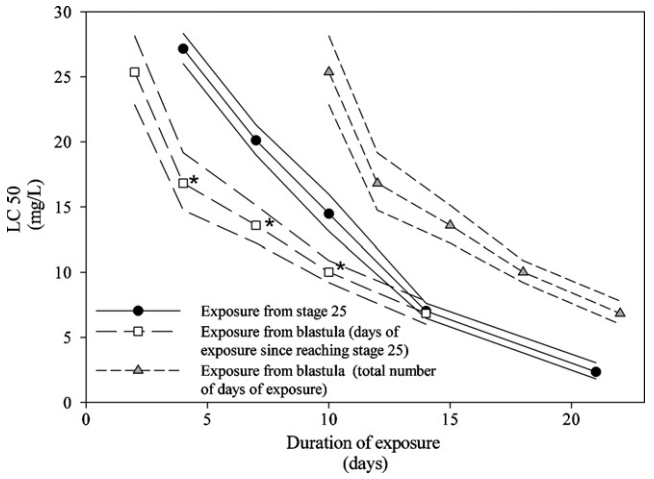

Fig. 3. Variation with exposure duration of lethal concentration 50 (LC50) in *R. arenarum* exposed to atrazine from either stage 4 or stage 25. Exposure from stage 4 is illustrated both in terms of the total number of days since the beginning of exposure and the number of days since reaching stage 25. Lines on each side of the plots represent the 95% confidence intervals. * = significantly different from the LC50 obtained after the same duration in exposures from stage 25 ($p < 0.05$).

higher doses in a number of amphibian species, a second set of exposures starting from stage 25 was conducted using a range of concentrations extending four orders of magnitude below the lowest concentration used in the first set of 21-day exposure (1 mg/L). Data from this second set of exposures are presented in Fig. 2. This figure shows that none of the concentrations tested below 1 mg/L caused significant mortality of the larvae during 21 days of exposure. In all experiments starting with premetamorphosis larvae, larvae of both controls and treated groups remained at stage 25–26 throughout the 21 days of exposure.

Interestingly, as embryos took 7–8 days to reach stage 25 in exposures from stage 4, the first significant mortality observed after 10 days of exposition in exposures from stage 4 is in fact equivalent to 2–3 days of exposure in stage 25, a timing similar to the first appearance of mortality in exposures from stage 25. Fig. 3 illustrates the greater similarity of the LC50s obtained after various durations in exposures from stage 4 and 25 when exposure duration is expressed in terms of the time since reaching stage 25 rather than the total number of days in exposures from stage 4. However, although the LC50s are more similar when exposure from stage 4 is expressed in terms of the number of days since reaching stage 25, the values of LC50 remain significantly different until reaching 14 days of exposure, when differences no longer exist.

3.2. Exposure from prometamorphosis until completion of metamorphosis

Survival curves of the larvae exposed to atrazine from stage 38–39 until the completion of metamorphosis are shown in Fig. 4. This figure shows that no larvae survived treatment with 15 and 20 mg/L of atrazine, and less than 50% of the animals survived when exposed to 10 mg/L of atrazine. Concentrations of atrazine of 5 mg/L and lower did not significantly affect survival (Fig. 4). A comparison of the LC50s obtained for the exposures starting from stage 25

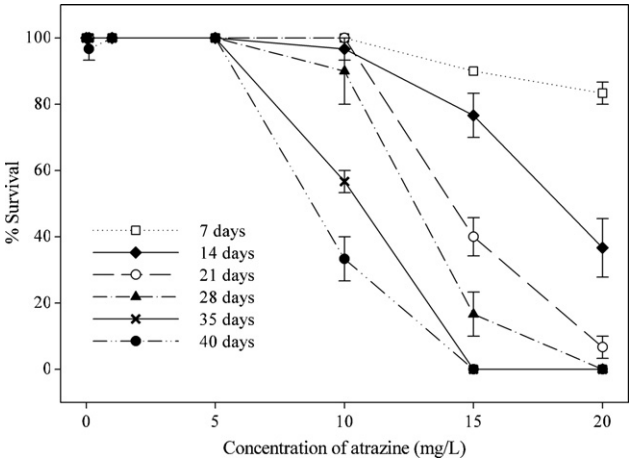

Fig. 4. Concentration–response curves describing the survival of larvae of *R. arenarum* as a function of atrazine concentration when exposed from stage 38–39 while completing metamorphosis.

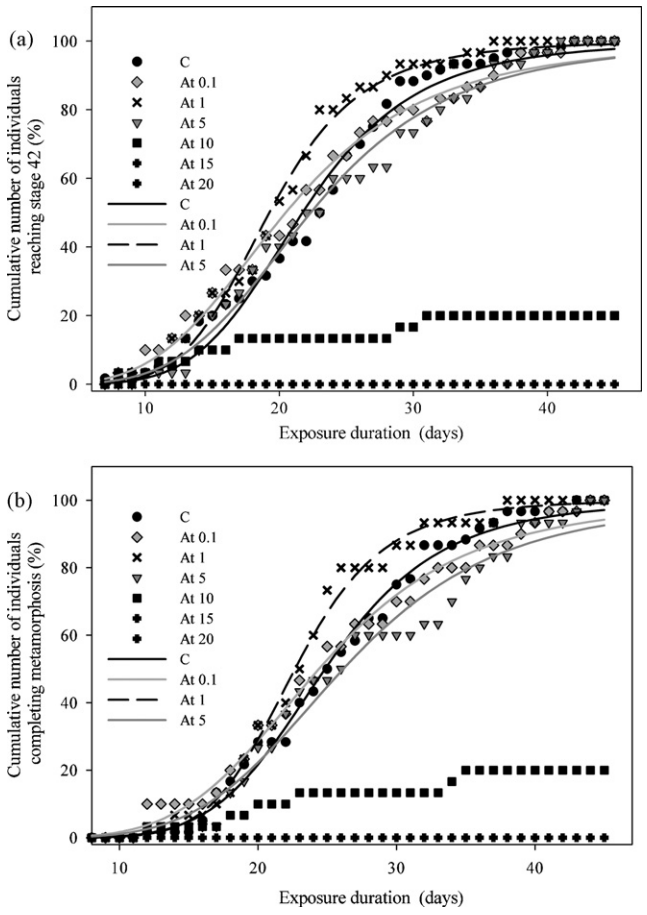

Fig. 5. Cumulative number of individuals (a) reaching stage 42 (all four legs emerged) and (b) completing metamorphosis (complete tail resorption) over time for controls (C) and larvae exposed to atrazine. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in mg/L. The lines represent the sigmoidal curve models calculated for treatments where 100% of the animals reached stage 42 and completed metamorphosis. Error bars are omitted to reduce clutter.

and stage 38–39 reveals a greater sensitivity to atrazine of larvae in stage 25. Indeed, the LC50s obtained for larvae in stage 25 equaled 7.03, and 2.36 mg/L after 14 and 21 days of exposure, respectively, whereas corresponding values for larvae in stage 38–39 were 18.27 and 14.43 mg/L (Table 2). Oppositely, larvae in stage 38–39 were shown to be more sensitive to atrazine than embryonic stages when comparing survival after 7 days of exposure to 20 mg/L of atrazine. Such an exposure resulted in 96.7% of survival of embryos in stage 4 whereas only 83.3% of larvae in stage 38–39 survived ($p = 0.048$, Mann–Whitney Rank sum test). Exposures from stage 4 longer than 7 days cannot be used to compare sensitivity of embryonic stages, as embryos eventually transform into stage 25 larvae in longer exposures.

The cumulative number of animals reaching stage 42 and completing metamorphosis over time is shown in Fig. 5a and b, respectively. This figure shows that none of the animals exposed to 15 and 20 mg/L of atrazine reached stage 42 nor completed metamorphosis. As mentioned above, these concentrations eventually resulted in all animals dying. With 10 mg/L of atrazine, only 20% of the larvae reached stage 42 and completed metamorphosis, most of them in the first 22 days of exposure, as mortality increased rapidly afterwards and only 33.3% of animals survived the exposure (Fig. 4). All animals reached stage 42 and completed metamorphosis with exposures to 5 mg/L or less of atrazine. It took 33.3 and 36.4 days for 90% of the animals from the control group to reach stage 42 or complete metamorphosis, respectively.

Looking at Fig. 5, larvae exposed to 1 mg/L of atrazine appear to reach stage 42 and metamorphose faster than larvae from the control group or larvae treated with 0.1 or 5 mg/L of atrazine, while the curves describing the metamorphosis of these last treatments also appear to present differences between each other. These apparent variations in the timing of the completion of metamorphosis between the various treatments were statistically evaluated by comparing the time necessary for 50% of the individuals to reach stage 42 (T42) or complete metamorphosis (TCM). The values obtained for the various treatments are presented in Fig. 6a. This figure shows that T42 was significantly accelerated by atrazine and that the concentration–response relationship was U-shaped, with 0.1 and 1 mg/L of atrazine gradually reducing T42 while 5 mg/L had no significant effect.

The effect of atrazine on TCM was distinct from that observed on T42 with no significant differences with controls being observed with 0.1 mg/L of atrazine, while 1 mg/L of atrazine significantly decreased and 5 mg/L significantly extended TCM (Fig. 6a). The disparity in the effect of atrazine on T42 and TCM can be better understood if the time interval between T42 and TCM is considered. Values obtained for this parameter are shown in Fig. 6b. It can be seen from this figure that treatment with 0.1 and 5 mg/L of atrazine significantly increased the interval between T42 and TCM compared to controls. A similar tendency for a dilatation of the

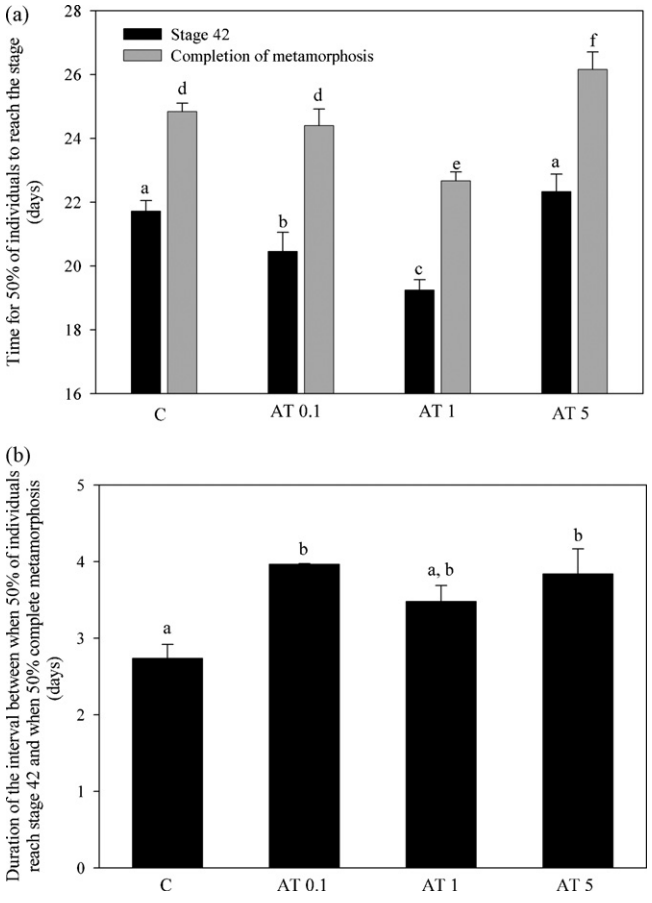

Fig. 6. (a) Time required (mean ± S.E.) for 50% of the larvae of *R. arenarum* to reach stage 42 and complete metamorphosis when exposed to atrazine. (b) Duration of the interval (mean ± S.E.) between when 50% of *R. arenarum* larvae reach stage 42 and when 50% complete metamorphosis when exposed to atrazine. At 0.1 = 0.1 mg/L of atrazine, At 1 = 1 mg/L of atrazine, and At 5 = 5 mg/L of atrazine. Controls are denoted by C. Bars with the same letter are not significantly different ($p < 0.05$).

interval between T42 and TCM is also present at 1 mg/L of atrazine but the difference with the control group is not statistically significant. Taking into account the effects of atrazine on both T42 and on the interval between T42 and TCM, it is possible to understand its effect on TCM. With 0.1 mg/L of atrazine, the diminution of T42 is compensated by the dilatation of the interval between T42 and TCM and for this reason TCM is not significantly different from controls. With 1 mg/L of atrazine, the interval between T42 and TCM is only slightly increased and does not compensate the large reduction of T42, resulting in a reduced TCM compared to controls. Finally, at 5 mg/L of atrazine, the absence of an effect on T42 combined with an increase of the interval between T42 and TCM results in a TCM that is greater than controls.

4. Discussion

Exposures to atrazine realized in the present study with various embryo-larval stages of *R. arenarum* revealed that larvae in premetamorphosis are more sensitive than larvae in prometamorphosis and that these are, in turn, more sensitive than embryonic stages. Indeed, concentrations of atrazine as high as 30 mg/L had little effects on embryonic stages, the embryos surviving and developing in a similar manner as controls. Results from Birge et al. (2000) suggest that the sensitivity of early embryonic stages is variable amongst anuran species as LC50 values ranging between 0.41 and 127 mg/L were obtained when embryos of various species were exposed to atrazine for 7–12 days from fertilization. With a LC50 value of 25.37 mg/L when exposed for 10 days from stage 4, *R. arenarum* embryos would seem to be at an intermediate position within the spectrum of sensitivities.

Furthermore, embryos of *R. arenarum* appear to be less susceptible to malformations caused by atrazine compared to the few other species for which data are available. Indeed, whereas concentrations of atrazine as high as 30 mg/L did not significantly increase the incidence of malformations in the current study, Allran and Karasov (2001) observed a dose-dependent increase in deformed larvae when exposing *R. pipiens*, *Rana sylvatica* and *B. americanus* to concentrations of atrazine ranging between 0 and 20 mg/L. Embryos of *X. laevis* were also more prone to malformations than *R. arenarum*, exposure to 8 mg/L of atrazine in natural waters causing malformations in 100% of individuals (Morgan et al., 1996).

Of the three developmental stages of *R. arenarum* tested in the current study, stage 25 was the most sensitive and, in exposures from stage 4, mortality was not observed until the embryos transformed into stage 25 larvae. However, once embryos exposed from stage 4 reached stage 25, lethality was greater over their first 10 days as stage 25 larvae when compared to the lethality observed in larvae first exposed to atrazine at stage 25 (Fig. 3). This observation denotes a sensitization of the larvae by previous embryonic exposure, which might be related to the potential for bioconcentration of atrazine previously described for amphibians (Allran and Karasov, 2000; Edginton and Rouleau, 2005).

The 96-h LC50 value of 27.16 mg/L obtained in the current study for stage 25 larvae is comparable to the values of 26.5 and 37.6 mg/L reported by Howe et al. (1998) for stage 29 larvae of *B. americanus* and *R. pipiens*, respectively. However, in contradiction with the current study, these authors noted an increase in sensitivity to atrazine as the larvae reached later stages; the 96-h LC50 decreasing to 10.7 and 14.5 mg/L in stage 40 *B. americanus* and *R. pipiens*, respectively. As mentioned above, in the present study, tadpoles of *R. arenarum* were found to be, on the contrary, less sensitive to atrazine at stage 38–39 than at stage 25.

Results from the current study also differ from results obtained by Storrs and Kiesecker (2004), which observed significantly lower survival with 0.003 mg/L of atrazine than with either 0.03 or 0.1 mg/L in early and late stage larvae of *Pseudacris crucifer* and *R. clamitans*, as well as in early but not late stage larvae of *B. americanus*. In contrast, in the present study, concentrations of atrazine between 0.0001 and 0.1 mg/L did not significantly affect survival of stage 25 larvae of *R. arenarum*, during 21 days of exposure. Significant mortality first appeared with 1 mg/L of atrazine (Fig. 2) and a monotonous concentration–response relationship was thereafter present with increasing concentrations of atrazine (Fig. 1b). The differences observed between the current study and the two studies above mentioned are possibly due to the fact that this study used technical-grade atrazine with a purity of 98% while the other two studies used commercial preparations containing only 85% and 40.8% of atrazine. The remaining components of the field-grade formulations will, indeed, possess their own toxicity and may have altered the survivorship patterns. In the light of the current study, it, therefore, remains to be proven whether the non-monotonous survival dose–response curve observed by Storrs and Kiesecker (2004) is really caused by atrazine or if it is due to other components of the formulation.

In larvae of *R. arenarum* having reached stage 38–39, exposition to concentrations of atrazine of 10 mg/L and above widely prevented completion of metamorphosis and caused important mortality. On the other hand, whereas 100% of animals eventually completed metamorphosis when exposed to concentrations of atrazine between 0.1 and 5 mg/L, the timings of metamorphosis were altered from 0.1 mg/L, the lowest concentration tested. Indeed, results obtained demonstrated that concentrations of atrazine between 0.1 and 5 mg/L decreased the time needed for 50% of the larvae to reach stage 42 (four limbs and tail present) and that the relationship describing this response was a non-monotonic U-shaped dose–response curve (Fig. 6a). Furthermore, it was also found that the same range of atrazine concentrations significantly extended the duration of the process of tail resorption, which corresponds to the interval between stage 42 and the completion of metamorphosis. The amplitude of the lengthening of the time needed for tail resorption was independent of concentration and was similar between 0.1 and 5 mg/L of atrazine. Overall, the combination of these two different facets of atrazine influence on metamorphosis resulted in a significant acceleration of metamorphosis at 1 mg/L and a significant increase in the duration of metamorphosis at 5 mg/L, whereas no significant difference was observed with 0.1 mg/L.

Although it has never been clearly stated in the scientific literature that atrazine can cause non-monotonous accelerations or decelerations in the time needed for completing metamorphosis, a number of studies have provided data supporting such an interpretation (Diana et al., 2000; Coady et al., 2004; Freeman et al., 2005). In particular, using another species of Bufonidae, Freeman et al. (2005) also observed an acceleration of metamorphosis which, similar to the current study, presented a non-monotonic dose–response; the acceleration of metamorphosis being of a greater amplitude at 0.25, than at 0.5 and 1 mg/L whereas no difference existed between controls and larvae exposed to 5 and 10 mg/L (Freeman et al., 2005). The coherence of the results obtained by the current study and the ones of Freeman et al. (2005) highlights the capacity for atrazine to accelerate anuran metamorphosis in a U-shaped fashion in at least some species. Although this study was not designed to examine potential mechanisms by which atrazine might influence metamorphosis, general hypotheses can be proposed regarding potential modes of action though which atrazine may hasten metamorphosis.

It is widely recognized that the thyroid hormones thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) are the causative factor governing anuran metamorphosis, and that addition of T_3 or T_4 to the rearing water can induce or speed up metamorphosis in a variety of species

(Shi, 2000). Similarly, it has long been established that, as is the case for mammals, thyrotropin (TSH) secreted by the pituitary gland is responsible for the induction of thyroid hormone release in anurans. However, in contrast with mammals and adult amphibians, amphibian larvae present the distinctive feature that hypothalamic control of TSH release is mediated through the corticotropin-releasing factor (CRF) instead of the thyrotropin-releasing factor (TRF), even though this last hormone is present during amphibian metamorphosis (Denver and Licht, 1989; Shi, 2000; Okada et al., 2004). This peculiarity of the hypothalamic–pituitary–thyroid (HPT) axis of larval amphibian is exemplified by the fact that administration of CRF accelerates metamorphosis in a number of anurans, including *R. arenarum* (Denver, 1993; Miranda et al., 2000).

Given that the more classical function of CRF, the stimulation of the hypothalamic–pituitary–interrenal axis (HPI) responsible for the secretion of glucocorticoids and the non-specific stress response, is also operational in larval amphibians, there results an intricate relationship between the two hormonal axes (Hayes, 2000). Indeed, this dual function of CRF means that non-specific stress can potentially stimulate the secretion of not only corticoids but also thyroid hormones. Glucocorticoids have, furthermore, been shown to interact with the HPT axis in several other ways. For example, glucocorticoids enhance thyroid hormone activity by increasing the binding of T3 to its receptor and accelerate the conversion of T4 to the more potent T3 (Hayes, 2000). A clear example of the interrelation existing between the general stress-response and the HPT axis lies in the observation that amphibian larvae, especially those that breed in temporary aquatic environments, often have the ability to accelerate metamorphosis in response to environmental stressors such as high densities, deteriorating water quality, predation and limited resource availability (Wilbur and Collins, 1973; Boorse and Denver, 2003).

Considering that, similar to what is observed in other vertebrates, the alteration of amphibian homeostasis by xenobiotics can induce a general stress response through the HPI axis (Gendron et al., 1997), a possible interpretation of the acceleration of metamorphosis observed in *R. arenarum* exposed to atrazine may be that, as Freeman et al. (2005) suggested for *B. americanus*, atrazine is acting as a typical environmental stressor promoting the acceleration of metamorphosis in order to escape the contaminated aquatic system. In this context, the presence of a U-shaped response of T42 to atrazine, would not be surprising as such a non-monotonic dose–response curve is frequently reported for stressors in a variety of biological systems (Calabrese et al., 2007; Rattan et al., 2007; Gems and Partridge, 2008), possibly due to the intrinsic properties of glucocorticoid receptor-mediated gene expression (Li et al., 2007).

Alternatively, alterations in hypothalamic monoamine systems and the resulting modification of pituitary hormone secretion have been suggested as the primary site of action of atrazine in rats, resulting in accelerated aging and premature appearance of mammary gland tumors in females (Cooper et al., 2007), while causing delayed pubertal progression and reproductive tract development in males (Stoker et al., 2002). Given that the pituitary of amphibian adults and larvae is also innervated and controlled by monoaminergic nerves (Enemar et al., 1967; Terlou and van Straaten, 1974; Aronsson, 1976), it is plausible that the influence of atrazine on metamorphosis is due to a similar mode of action.

Interestingly, in rats, the hormonal alterations that result from the neurotoxicity of atrazine and which mediate its reproductive effects include an elevation of prolactin concentrations (Stoker et al., 2002). In amphibian larvae, prolactin, the secretion of which is also under both inhibitory and stimulatory regulation by the hypothalamus, has emerged as a hormone that regulates metamorphic changes (Shi, 2000). Furthermore, Huang and Brown (2000) showed that transgenic *X. laevis* overexpressing the prolactin gene exhibit an adult-shaped body with an unabsorbed tail. In view of these findings, it appears possible that the increase in the time needed for tail resorption (i.e., the interval between T42 and TCM) observed in the current study in *R. arenarum* was associated to an effect of atrazine on prolactin secretion.

Regarding the ecological significance of the alterations in TCM that were observed in the current study, these may seem insignificant if it is considered that there is only 2-day difference between controls and animals exposed to 1 or 5 mg/L of atrazine. However, given that this 2-day difference originates from a disparity in the slopes of the curves representing the cumulative number of animals completing metamorphosis, this difference translates into a 4- and 6-day difference, respectively, when considering the time needed for 80 and 90% of animals to complete metamorphosis. This means that, with 1 mg/L of atrazine, 90% of completion of metamorphosis will be reached 6 days earlier than when atrazine is absent, whereas with 5 mg/L of atrazine this same percentage of completion of metamorphosis will occur 6 days later than when atrazine is absent.

In a pampean temporary pond typical of where *R. arenarum* larvae normally develop, water levels can change considerably in 2–6 days and the pond may even dry out (Natale, 2006). The acceleration of metamorphosis caused by 1 mg/L of atrazine could, therefore, seem, on first approach, to favor survival of a greater number of metamorphs while 5 mg/L of atrazine would appear to have an opposite effect. However, before reaching such a conclusion, it would be essential to examine better features of the metamorphs such as size at metamorphosis and physiological integrity, as a smaller and unhealthy metamorph would present reduced fitness. More importantly, the fact that alterations in the timing of metamorphosis were observed, in the current study, after exposure to a relatively low concentrations of atrazine over only the last part of the metamorphic process calls for more research to be done. Indeed, given the results obtained in this study, it would appear important to determine whether more important effects can be detected in longer exposures to lower concentrations of atrazine.

In conclusion, of the three developmental stages examined, premetamorphosis larvae (stage 25) were the most sensitive to atrazine. Still, the LC50s calculated for these larvae at various time intervals were all considerably elevated compared to the concentrations of atrazine usually observed in aquatic environments. Normally, this finding would suggest it unlikely for atrazine to cause direct mortality to *R. arenarum* under typical conditions of use. However, in this case, it will remain impossible to rule out the possibility of direct lethality until it is examined whether the failure to observe a pattern of greater mortality at lower concentrations as reported by Storrs and Kiesecker (2004) was due to a specific attribute of *R. arenarum* or whether it was due to the fact that a technical-grade atrazine was used instead of a formulated product. Solving this question is of prime importance as much for *R. arenarum* as for other species given that atrazine is typically applied to crops as a formulated product.

In terms of sublethal effects, an alteration of the timings of metamorphosis was observed starting from 0.1 mg/L, the lowest concentration tested in the current study. Essentially, larvae exposed to 0.1 and 1 mg/L of atrazine reached climax a few days earlier than controls and afterwards took more time for tail resorption whereas larvae exposed to 5 mg/L of atrazine reach climax in an interval similar to controls but also showed a delay in tail resorption. The consequences of these effects were that 1 mg/L of atrazine allowed metamorphs to escape the aquatic environment earlier than controls whereas 5 mg/L extended the time needed for the larvae to complete metamorphosis. More research is needed before the ecological significance of these findings can be fully understood.

Acknowledgments

Funding for this work was provided by the "Consejo Nacional de Investigaciones Científicas y Técnicas" (CONICET PIP 5324) and the "Agencia Nacional de Promoción Científica y Técnica" (PICT 14375). We wish to acknowledge Dr. Guillermo S. Natale for his help with the determinations of atrazine in water.

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